by Hammes et al. that only about 1% of the ^{3}H-label in their experiments is associated with the cells is difficult to interpret.

Although some of the experiments by Hammes et al. are consistent with their "bound steroid" hypothesis (see, for example, Figure 2A), others are not (for example, see Figures 2B and 2C). At a concentration of 1 nM DHT plus 1 nM SHBG, 50% of the steroid exists in the free form. One nanomole of DHT-SHBG and 1 nM DHT are equally effective at entering cells and activating an androgen-responsive reporter gene (Figure 2B); SHBG alone is about 25% as effective at activating the reporter gene as DHT alone. The most reasonable interpretation is that free DHT is the active species and that SHBG itself has a modest effect (through mechanisms not explained by these experiments). RAP almost completely inhibits activation of the reporter gene by 1 nM DHT-SHBG, despite the fact that half of the DHT is unbound at this concentration. In contrast, 1 nM DHT alone is not blocked by RAP and yields the same increase in reporter gene activation as 1 nM DHT-SHBG. It is clear that SHBG is not necessary for DHT to enter the cell and to activate the reporter gene.

Thus, the conclusion by Hammes et al. that there is a megalin-mediated endocytic pathway for the uptake of androgens and estrogens bound to their SHBG proteins does not seem to be sufficiently supported by the evidence presented.

Note

All percentages for free steroids are calculated from the law of mass action for steroid protein interactions using the equations set out by Sodergard et al. (1982). The dissociation constants (Kd) used in the calculations are 1 nM for the binding of testosterone to SHBG, and 0.5 nM for the binding of DHT to SHBG.

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Response: Cellular Uptake of Sex Steroid Hormones

In our study (Hammes et al., 2005), we set out to understand the role of megalin, an endocytic receptor of the LDL receptor gene family, in reproductive tissues. We chose the most appropriate experimental models available to us: a megalin knockout mouse and the BN16 cell line, which has been widely used to study megalin-mediated endocytosis.

In the megalin knockout mouse, we showed that a deficiency in the megalin receptor resulted in the impaired development of male and female reproductive tissues and in sex steroid insensitivity, implicating megalin in androgen and estrogen action in vivo. In BN16 cells, we showed that megalin, an established endocytic receptor for the carrier bound steroid hormone 25-hydroxyvitamin D_3, can mediate the uptake of sex steroids bound to physiological carriers such as sex hormone binding globulin (SHBG) and androgen binding protein. These findings suggest that sex steroids not only enter cells by free diffusion but may also use endocytic pathways to gain entry into certain target cells. Furthermore, these data provide a mechanistic basis that can explain the observed sex steroid insensitivity in the megalin-deficient mouse.

Megalin knockout mice suffer from obstruction of the vaginal cavity in females and impaired descent of the testes in males, albeit at normal plasma levels of sex steroids (Table 1, page 755 of Hammes et al., 2005). These phenotypes are reminiscent of those found in animals treated with androgen and estrogen receptor antagonists (Hammes et al., 2005). Contrary to Dr. Rosner's claim, this phenotype cannot be accounted for by vitamin D deficiency because animals lacking the vitamin D receptor exhibit very low levels of estrogens and, as a consequence, show uterine hypoplasia and impaired sperm motility (Kinuta et al., 2000), which are defects not seen in megalin knockout mice.

We agree that little SHBG is found in adult mouse plasma, suggesting that this carrier protein has little relevance for systemic transport of sex steroids in adult rodents; the situation may be different during development when

SHBG is produced by the fetal liver (Sullivan et al., 1991). However, as Dr. Rosner (Kahn et al., 2002) and others have reported, SHBG is expressed in adult steroid target tissues where it has been implicated in local hormone action. This is in agreement with our model of a role for megalin in mediating specific actions of androgens and estrogens (testicular descent, opening of the vaginal cavity) but not in the mediation of their systemic functions. Our data do not exclude roles for other steroid carriers in the endocytic delivery of sex steroids, such as α-fetoprotein or albumin, that also bind to the multiligand receptor megalin (Cui et al., 1996). The fact that BN16 cells do not express nuclear hormone receptors is irrelevant for our study because we used these cells to investigate the route of cellular uptake of bound sex steroids. We determined binding to the cells and internalization, which precede, and are independent of, the subsequent signaling pathways activated by sex steroid hormones.

Dr. Rosner also raises issues concerning technical details of our cell culture studies. Concerning the specificity of the megalin-SHBG interaction, we fail to see how the point regarding uptake studies in cells using denatured proteins could impact our interpretation of the biological role of the native protein. We have extensively confirmed the specificity of SHBG binding to megalin using state-of-the-art approaches, such as surface plasmon resonance analysis (Hammes et al., 2005; Figure 1A), inhibition with antagonists (Figure 1B) and α-megalin antisera (Figure S3), competition with excess ligand (Figure S3), and blockade of lysosomal degradation.

With respect to the kinetics of steroid uptake, Dr. Rosner inappropriately compares 40% degradation of ^{125}I-SHBG (Figure 1B) with 11% ^{3}H-testosterone being associated with cells (Figure 1C), interpreting this discrepancy as evidence against uptake of steroid and carrier in a complex. In Figure 1B, we use degradation assays to evaluate the amount of ^{125}I-SHBG that passes through the endocytic compartments in a 5 hr period by determining lysosomal degradation products accumulating in the cell medium. In Figure 1C, we measure the amount of ^{3}H-testosterone associated with cells at a given time, disregarding tracer that has passed through the cells earlier and that was expelled again. The experiment that Dr. Rosner requests is shown in Figure 1E where we label preformed complexes (either the steroid or the carrier moiety) and show that identical amounts (approximately 7%) are associated with cells.

Whether or not steroids that have been taken up by BN16 cells through megalin are subsequently chemically modified is irrelevant to our investigations as we merely quantify the amount of steroid present in cells at a given time point in the absence or presence of carriers or megalin antagonists. Any cellular activity following steroid internalization (such as modification) is clearly dependent on the enzymatic repertoire of individual cell lines and is secondary to the uptake process. Remarkably, in his own studies on the role of SHBG in the cellular uptake of ^{3}H-androgens, Dr. Rosner also determined cell-associated radioactivity but not enzymatic modification of the steroids (Plymate et al., 1991). The experiments measuring fractional uptake of ^{3}H-testosterone/SHBG complexes in the presence of excess SHBG, requested by Dr. Rosner, are shown in Supplemental Figure 3 (Figure S3).

In Figure 2, Dr. Rosner is misinterpreting the experimental design that underlies ligand-carrier complex formation. In panel A, purified steroid/SHBG complexes were used. In panels B and C, 1 nM testosterone was mixed with a large excess of SHBG from conditioned media as stated in the figure legend. Thus, more than 95% (not 50%) of the steroid is bound under these conditions. In conclusion, our cell studies reaffirm that complexed steroids can enter cells by receptor-mediated endocytosis of SHBG. Our findings directly support the work of Dr. Rosner, who, for more than 20 years, has championed the concept of cell-surface receptors for SHBG on steroid target cells that play roles in local actions of sex steroids bound to SHBG.

Contrary to Dr. Rosner's statement, we do not claim that steroids cannot enter cells by free diffusion in vitro or in vivo (free hormone hypothesis). Rather, we suggest that specific, spatially and temporally defined, actions of sex steroids (such as maturation of the reproductive organs) may be critically dependent upon active transport and selective concentration of the hormone by receptor-mediated endocytosis, rather than relying on passive diffusion.

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